Rec'd PCT/PTC 22 AUG 2000
PCT/DE98/03818

Novel sequence variants of the human beta2-adrenergic receptor gene and use thereof

The invention relates to novel sequence variants of the human beta2-adrenergic receptor gene and to their use for diagnosing a range of diseases, in particular for detecting dispositions to high blood pressure and for developing therapeutic agents on the basis of pharmacogenetic principles.

The human beta2-adrenergic receptor is an important component of the sympathetic nervous system, regulating as such a range of central and peripheral functions such as cardiovascular functions, metabolic functions, central nervous functions and neurosecretion. It is the point of attack for pharmaceutical/therapeutic agents with a broad range of indication belonging to drugs administered most frequently. Manifold findings point to the fact that this receptor might play a part in the pathogenesis/pathophysiology of a number of frequent diseases such as e.g. hypertension and other cardiovascular diseases, various neuropsychiatric diseases such as e.g. depression and metabolic diseases such as e.g. obesity (Insel PA (Ed) (1987) Adrenergic receptors in man, Marcel Dekker, New York, Basel).
The invention aims at detecting variants, polymorphisms, mutations and resulting haplotypes in the DNA sequence of the human beta2-adrenergic receptor gene and their correlations with the dispositions to diseases. Proceeding from these correlations a method for diagnosing these dispositions to diseases, for predicting the degree of severity, the course and survival time, a system for predicting the individual responsiveness to beta2 active therapeutic agents, for developing individual specific beta2 receptor agonists and antagonists and a system for developing a new class of beta2 effective therapeutic agents and for developing test systems for the investigation of pathophysiological connections and for developing the above-mentioned therapeutic agents. To sum up it is possible to predict or develop an individually optimum therapeutic agent for each beta2 genotype. The task is solved according to the claims, the subclaims are preferential variants.

It was stated that in the 5'-regulating region of the sequence of the human beta2-adrenergic receptor gene further variants are present, apart from the 3 mutations already known in the coding region (in positions 1633, 1666 and 2078). Furthermore, there was detected that these genetic variants correlate with the disposition to various diseases, e.g. high blood pressure.

Accordingly, the object of the invention is the sequence of the human beta2-adrenergic receptor gene which is entirely or partly mutated in the positions 159, 245, 565, 934, 1120, 1221, 1541, 1568, 1633, 1666, 1839, 2078, 2110, 2640 and 2826. In particular, a sequence containing entirely or partially the mutations T->A (position 159), A->G (position 245), G->A (position 565), G->A (position 934), G-> C (position 1120), C->T (position 1221), C->T (Arg->Cys) position 1541), T->C (position 1668), A->G (Arg-> Gly) (position 1633), C->G (Gln->Glu) (position 1666), G->A (position 1839), C->T (Thr-> Ile) (position 2078), C->A (position 2110), G-> C (position 2640) and G-> A (position 2826) (Figs. 1, 2a and 2b) is concerned.

Especially important are the following sequences (haplotypes):

- sequence with the mutations 1541, 1633 A and 1666 C,
- sequence with the mutations 1541 C, 1633 G and 1666 G,
- sequence with the mutations 1541 T, 1633 G and 1666 C,
- sequence with the mutations 1541 T, 1568 T, 1633 A and 1666 C,
- sequence with the mutations 1541 C, 1568 C, 1633 G and 1666 G and
- sequence with the mutations 1541 T, 1568 T, 1633 G and 1666 C.

Furthermore, a method to determine dispositions to diseases is the object of the invention where all sequences and variants of the beta2-adrenergic receptor gene of the individual mutation up to any potential combinations of all variants (including any absolute number of variants which may be included) may be genotypified, allowing to furnish respective data on dispositions to diseases.

The method is characterized by the fact that the DNA of a proband is isolated and genotypified at least in one of the positions exchanged and subsequently compared with the reference DNA sequence. Forms where at least position 1633, at least the three positions 1541, 1633 and 1666 or the four last-mentioned positions (1541, 1568, 1633 and 1666) or the seven positions 245, 565, 934, 11541, 1568, 1633 and 1666 are genotypified.

The method may be also varied by genotypifying at least 3 of the 4 positions 1541, 1568, 1633 and 1666 and subsequently comparing them with the reference DNA sequence. Here, genotypifying of the positions 1541, 1633 and 1666 is preferred.

Genotypifying is carried out by sequencing or by means of other methods suited for the detection of point mutations. They involve PCR-aided genotypification methods such as e.g. allel-specific PCR, other genotypification methods using oligonucleotides (examples would be 'dot blotting' or 'oligonucleotide ligation assays' (OLA), methods using restriction enzymes and 'single nucleotide polymorphism ' (SNP) analysis by means of 'matrix-assisted laser desorption/ionization mass spectrometry (MALDI) and, in principle, any method to detect variants which will be available in future including chip technology in all its technological variants.

Proceeding on it, the method according to the present invention for determining a broad spectrum of most various dispositions to diseases is suited.

In a variant it is suited e.g. for the determination of high blood pressure (or for predicting the region of the individual high blood pressure values per se), and other cardiovascular diseases including myocardial infarct and apoplexy, in the widest sense the development of a terminal renal insufficiency (being in need of dialysis).

A further preferential variant allows e.g. to determine a disposition to neuropsychiatric diseases such as depressions and anxiety syndromes (anxiety disorders), attention deficit disorder (with hyperactivity), eating disorder, e.g. for anorexia nervosa and bulimia, or disorder caused by posttraumatic stress; or to diseases of the autonomic nervous system such as e.g. Bradbury-Eggelston, Sky-Drager and Riley-Day syndromes and selective noradrenergic and baroreceptor dispositions or migrain.

In addition, it is also suited for detecting dispositions to allergic diseases, in particular asthma and atopic disorder.

A further application is the determination of a disposition to metabolic diseases such as obesity (and family "morbid obesity") including a prediction of the weight area as such and a disposition to change of weight, finally a prediction of the proportion of the measurements of the body as such as they are e.g. expressed in the "body mass index" (BMI).

Furthermore, the method allows also the determination of the course and the severity of diseases and the prediction of survival after severe medical diseases, e.g. after myocardial infarct, cardiac failure and/or apoplexy.

A further preferential variant allows the determination of an individually varying reactivity of the autonomic nervous system, in particular to endogenous and exogenous stress (as it is, e. g. in particular, expressed by an individually varying disposition to high blood pressure and/or heart rate modifications (deflections) or individually differing blood pressure modifications as a result of endogenously or exogenously induced changes of the salt concentration in blood (individually varying sensitivity or resistance to salt) and, in the widest sense, also by the individually varying salt and water regulation or reverse resorption in the kidney (volume regulation connected with it).

A further important object of the invention is the use of the required sequence variants a) for predicting the individually varying responsiveness to therapeutics known so far (beta2 receptor ligands) and the individually varying responsiveness to the endogenous ligands adrenalin and noradrenalin; b) preferably for developing individually specific beta2 receptor agonists and antagonists; c) in particular, also for developing a new class of therapeutics directed to the beta2 receptor gene which attack at the 5'regulatory region, promoter region, in particular e. g. at the leader peptide, and have effect via regulation of transcription, translation or affecting its efficiency, in particular by regulating the expression.
In this connection, a further object of the invention is predicting the individual habituation to the administration of pharmaceutical agents (tachyphylaxis) and a various disposition to side effects of pharmaceutical agents. Altogether, a prediction of individually optimum therapeutics on which various effective mechanisms are based is possible.

A further important object of the invention is the use of the claimed sequence variants for building up genes or vectors, in particular for the development of pharmaceutically relevant substances and for the development of a diagnostic kit or any diagnostic method. Such kits and methods may be used with a favourable effect for predicting the individual disposition to diseases or the individual responsiveness to various beta2-therapeutics.

Thus, cultures (cells) expressing the most various combinations of individual ß2-variants mentioned may serve as test models for the development of individual specific therapeutics (ß2-agonists and antagonists and beta2-expression regulating DNA therapeutics). This corresponds to test models *in vitro*, yet also *in vivo* test models are included (transgenic animals bearing these individual receptor variants).

As individual test models they allow in vitro (= ex vivo) a prediction of the individual functional state of the beta 2-receptor or the functions mediated by it.

The extent of the claimed invention is represented in detail hereinafter. To prepare the invention the whole DNA sequence known of the human beta2-adrenergic receptor gene including its regulating and coding regions in patients and checks by means of 'multiplex PCR sequencing' are investigated and, first of all, a number of genetic variants is identified. In the 5' regulating region eight new variants have so far been detected the most important of which seems to be the substitution of a highly preserved Arg->Cys in the 'leader peptide' of the gene (position 1541) which regulates the translation of the receptor gene (position –47 in relation to the starting point of translation), i. e. its expression.

Summary of the newly identified variants (nucleotide position before the substitution is related to the beta2-receptor gene sequence published (Koliba B.K. et al., Proc. Natl. Acad. Sci USA; 84(1): 46-50 (1987) [Acc. No. JO2960]; the information in brackets behind the substitution refers to the start of translation):

159 T -> A (-1429)
245 A -> G (-1343)
565 G -> A (-1023)
934 G -> A –654)
1120 G -> C (-468)
1221 C -> T (-367)
1541 C -> T (-47) Arg -> Cys substitution in the 'leader peptide' of the beta2 receptor gene
1568 T -> C (-20)

These variants are clearly represented in Figs. 1, 2a and 2b.

Correlations with diseases or clinically relevant phenotypes:

Specific effects of the two mutations known so far Arg -> Gly (in position +46 related to the starting point of translation, corresponds to position 16 of the amino acid sequence) and Gln -> Glu (in position +79 related to the starting point of translation, corresponds to position 27 of the amino acid sequence) and the newly detected 'leader peptide' mutation Arg -> Cys (in position –47 related to the starting point of translation) on a number of clinically and pathogenically relevant phenotypes were detected in a few studies. Thus, a significant association of the alleles in position 16 of the amino acid sequence with the genetic predisposition to hypertonia and extremely deflected blood pressure values was detected. The three mutations described hereinafter have a significant effect on phenotypical parameters such as heart rate, noradrenalin concentrations, blood pressure modifications as a result of experimentally induced physical and mental stress, 'coping styles' and personality dimensions such as weight and change of weight. In particular, also an association of the 'leader peptide' mutation with hypertonia was shown. Furthermore, it was possible to establish a relation between beta2-agonist induced vasodilatation and beta2 receptor mutations, preferably in position 16 of the amino acid sequence, and a relation between beta2 receptor expression of individuals genotypified in fibroblast cultures and beta2 receptor mutations, preferably in position 16 of the amino acid sequence.

Detection of specific three-mutation combinations in positions (related to the beta 2-sequence published, Kobilka et al. 1987) 1541 C -> T ('leader peptide' mutation Arg -> Cys), 1633 A -> G (Arg -> Gly) and 1666 C -> G (Gln -> Glu):

combination 1 : 1541 T (Cys allel), 1633 A (Arg allel), 1666 C (Gln allel)

combination 2: 1541 C Arg allel), 1633 G (Gly allel), 1666 G (Glu allel)

combination 3: 1541 T (Cys allel), 1633 G (Gly allel, 1666 C (Gln allel)

These three specific combinations occur in 80 – 95 % of the population, they seem to be selected evolutionarily from the total number of combinations to be expected and represent various functional states of the human beta 2-adrenergic receptor on which the variability of physiological and pathophysiological functions is based. In particular, they are connected with an individually varying responsiveness to endogenous ligands such as adrenalin and noradrenalin and with a various therapeutical responsiveness to beta2 receptor agnostis and

antagonists which enables these 'combinations' to be a starting point for the development of an 'individually tailored pharmacotherapy'.

Detection of specific beta2 'haplotypes' consisting of four variants: in the positions (related to the beta2 sequence published, Kobilka et al. 1987) 1541 C -> T ('leader peptide' mutation Arg -> Cys), 1568 T -> C; 1633 A -> G (Arg -> Gly) and 1666 C -> G (Gln -> Glu):

Combination 1: 1541 (Cys allel), 1568 T, 1633 A (Arg allel), 1666 C (Gln allel)
Combination 2: 1541 C (Arg allel), 1568 C, 1633 G (Gly allel), 1666 G (Glu allel)
Combination 3: 1541 T (Cys allel), 1568 T, 1633 G (Gly allel), 1666 C Gln allel)

Combination 1 was observed significantly more frequently in individuals having an inclination towards hypertonia, thus representing a genetic risk factor.

Detection of specific beta2 'haplotypes' consisting of seven variants:
Considering all variants in calculations it was possible to extract 'haplotypes' consisting of seven variants (including the three mutations mentioned); the calculations were aimed at identifying 'haplotypes' from the entirety of the genome which were sufficient to distinguish between the patient group and the control group. A specific 'haplotype', combination 1, may be more frequently observed in the case of a genetic loading by hypertonia. This may be extended to other phenotypes.

Combination 1: 245 G, 565 G, 934 A, 1541 T (Cys allel), 1568 T, 1633 A (Arg allel), 1666 C (Gln allel)
Combination 2: 245 A, 565 A, 934 G, 1541 C (Arg allel), 1568 C, 1633 G (Gly allel), 1666 G (Glu allel)
Combination 3: 245 G, 565 G, 934 G, 1541 T (Cys allel), 1568 T, 1633 G (gly allel), 1666 C (Gln allel)

The "haplotypes" described last describe finally the real, total individual functional state of the receptor. The invention is based on the concept that the various functional (dysfunctional) receptor states are not based on individual mutations but are a result of the individual "polymorphic" overall gene sequence as a function determining unity.
Subsequently, the invention is explained in greater detail by an example.

Material and methods

The multiplex PCR sequencing method is applied for ascertaining the total polymorphic spectrum of the beta2 receptor gene. To this end, the overall promoter region known so far and the coding region are subdivided into eight fragments and amplified by means of PCR (see Fig. 1). These PCR fragments were pooled and sequenced simultaneously. The fragments of the termination reactions were separated on a sequence gel and transferred to a nylon membrane by means of direct transfer electrophoresis (DTE). The individual sequence leaders were successively decoded by successively hybriziding with specific oligonucleotides.

The specific conditions for the amplification were as follows:

Forward primer ADRBR-F1 with the sequence 5'-TATTGGCCAGGATCTTTTGCTTTCTAT-3' and backward primer ADRBR-R1 with the sequence 5'-TAACATTAAGAACATTTTGAAGC-3' were used for fragment I. Fragment II was amplified by means of the two primers ADRBR-F2: 5'-GCATACCCCCGCTCCAGATAAA-3' and ADRBR-R2: 5'-GCACGCACATACAGGCACAAATAC-3'. For fragment III it were two primers ADRBR-F3: 5'-GGCCGCGTTTCTGTGTTGG-3' and ADRBR-R3: 5'-AGTGCGTTCTGCCCGTTATGTG-3'. For fragment VIII the two primers ADRBR-F8: 5'-GGTACTGTGCCTAGCGATAAC-3' and ADRBR-R8: 5'-TAAAATACCCCGTGTGAGCAAATAAGAG-3' were used. The reaction conditions for these four fragments were as follows: 10 x PCR buffer (100 mM Tris HCl, 15 mM $MgCl_2$ x 6 H_2O, 500 mM KCl, pH 8.3), dNTP 2 mM, 30µM primer F, 30 µM primer R, 50 ng of genomic DNA and 5 U of a *Taq* DNA polymerase. All three fragments were amplified with the following temperature profile: 94° C 4 min; 35 cycles: 94°C 30 sec., 60°C 30 sec., 72 °C 1 min. and finally 72°C 10 min.

Fragment IV was amplified with the aid of the two primers ADRBR-F4: 5'-GGGGAGGGAAAGGGGAGGAG-3' and ADRBR-R4: 5'-CTGCCAGGCCCATGACCAGAT-3'. For fragment VII the primers ADRBR-F7: 5'-CTGGCTGCCCTTCTTCATCGTT-3' and ADRBR-R7: 5'-TACCCTAAGTTAAATAGTCTGTT-3' were used. The conditions for these two PCR reactions were as follows: 10 x PCR buffer (160 mM $(NH_4)_2SO_4$, 0.1 % of Tween-20, 500 mM KOH, pH), dNTP 2 mM, 30 µM primer F, 30 µM primer R, 50 ng of genomic DNA and 4 U of a mixture of *Taq* DNA polymerase and a thermostable inorganic pyrophosphatase of *thermus thermophilus*. Both fragments were amplified with the following temperature profile:

94°C 4 min.; 35 cycles: 94°C 30 sec., 66°C [fragment IV] or 60°C [fragment VII] 30 sec., 72°C 1 min. and finally 72°C 10 min.

Fragment V was amplified by means of the two primers ADRBR-F5: 5'-ATGCGCCGGACCACGAC-3' and ADRBR-R5: 5'-GTAGAAGGACACGATGGA-3', fragment VI was amplified with the two primers ADRBR-R6: 5'-GCTACTTTGCCATTACTTCACC-3' and ADRBR-R6: 5'-AAATCTGGGCTCCGGCAGTAGATAAG-3'. These two fragments were amplified by means of 'AmpliTaq gold kits' by Perkin Elmer. In these two fragments the temperature profile was as follows: 94°C 10 min., 35 cycles: 94°C 30 sec., 56 °C [fragment V] or 58°C [fragment VI] 30 sec., 72 °C 1 min. and finally 72°C 10 min.

Sub E cont.

Sequencing was carried out by means of the 'thermo sequenase cycle sequencing kit' by Amersham. The PCR primers described above were used as sequencing primers. Sequencing was carried out in four multiplex pools. Pool 1 contained the sequencing primers ADRBR-F1, ADRBR-F3, ADRBR-F5 and ADRBR-F7; pool 2 contained the sequencing primers ADRBR-R1, ADRBR-R3, ADRBR-R5 and ADRBR-R7. Fragments I, III, V and VII were inserted into the two sequencing pools. Yet, pool 3 contained the sequencing primers ADRBR-F2, F4, F6 and F8; pool 4 contained the sequencing primers ADRBR-R2, R4, R6 and R8. Fragments II, IV, VI and VIII were inserted into these two pools.

All PCR and sequencing reactions were carried out in a PTC 225 cycler of MJ Research.

The products of the sequencing reaction were separated on a 100 µm thick acryl amide gel (5% acryl amide, 7 M urea) and under standard DTE conditions (see Richterich and Church, 1993) transferred to a biodyne A membrane (Pall). Then, the membrane was hybrizided with ^{32}P-marked oligonucleotides and the individual sequence leaders were detected with the aid of a phospho fluorimager (Storm 860, Molecular Dynamics).

Literature:

Kobilka, B.K., Dixon R.A., Frielle T.; Dohlman H. G., Bolanowski M.A., Sigal I.S., Yang Feng T.L., Francke U., Caron M.G., Lefkowitz R.J.: cDNA for the beta 2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci USA*; 84 (1): 46-50 (1987).

Parola A. L. and Kobila B.K. The peptide product of a 5' leader cistron in the beta 2-adrenergic receptor mRNA inhibits receptor synthesis. *J Biol chem*. 269 (6): 4497-505 (1994).

Richterich P. and Church G.M.: DNA sequencing with direct transfer electrophoresis and nonradioactive detection. *Methods Enzymol*. 218: 187-222 (1993).

Legends relating to the Figures:

Fig. 1

Polymorphic spectrum of the human beta 2-adrenergic receptor gene
Variants are indicated according to their nucleotide positions.
(Reference sequence Kobilka et al. 1987).

Fig. 2a

Sequence of the human beta 2-adrenergic receptor (Kobilka et al. 1987)
Variants are indicated according to their positions.

Fig. 2b

Sequence of the human beta 2-adrenergic receptor (Kobilka et al. 1997).
The variants (nucleotide or amino acid substitution) are indicated.